THINKING OUTSIDE THE DOT:
THE POWER OF IMAGING COMES TO FLOW CYTOMETRY.

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For more than a decade, technology for the quantitative analysis of digital cell images has developed closely in parallel with computing power. As computer processing speed and storage capacity have increased, so has the ability of a conventional desktop computer to perform calculations with the extremely complex data format presented by a living cell. Today, these common and relatively inexpensive computers can support the analysis of large numbers of cells in considerable detail and at high speeds.

The large majority of systems for imaging and analyzing cells have been designed to work with so-called 'adherent' cells – cells that grow naturally on solid surfaces such as glass slides, cover slips and multiwell culture plates. With the introduction of the ImageStream® system from Amnis Corporation, the ability to generate and quantify digital cell images has now been extended to include non-adherent cells, the most important of which are the various cell types in blood.

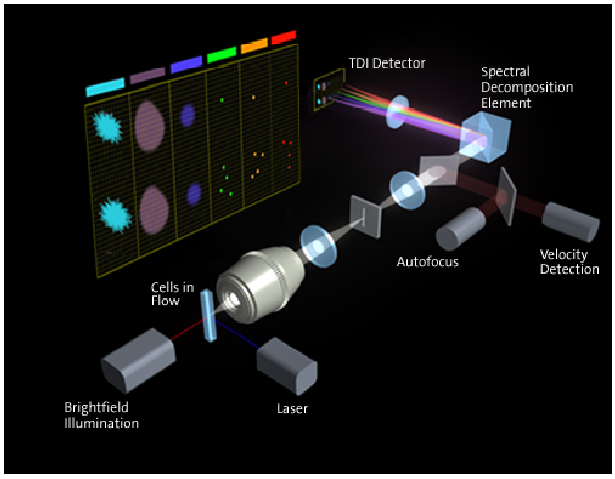
The ImageStream System

The ImageStream system represents the synthesis of two prior technologies: flow cytometry and microscopy. Flow cytometry, invented by Leonard Hertzenberg over 30 years ago, has become a technical mainstay in the fields of hematology and immunology. It is one of the most important technologies for studying AIDS and is used extensively in AIDS drug discovery. Standard flow cytometry of this kind does not, however, offer the capability of imaging cells, and while several attempts have been made over the years to achieve this valuable goal, they have been hindered by the long times required to capture fluorescent images. Flow cytometry and most forms of digital cell image analysis depend on the use of fluorescent probes that identify specific molecules on, or in, the target cell. When irradiated by lasers, these probes emit fluorescent light of highly defined wavelengths, but light that is typically too dim to image with a short exposure.

Microscopy, of course, is by far the most important tool in cell biology. Powerful optical systems can generate extraordinarily detailed images of cells, including fluorescent images. Yet a microscope yields information only one image at a time, and fully understanding a cellular phenomenon often depends on information not only about individual cells, but also about the entire population of cells – precisely the kind of information flow cytometry can provide.

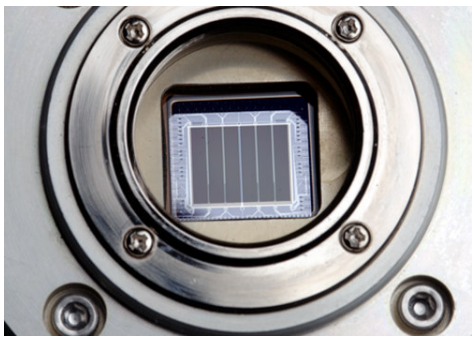
Flow cytometry offers extensive statistical information about cell populations; microscopy offers very detailed information about individual cell structures. By combining these two fundamental technologies into a single instrument platform, the ImageStream enhances both technologies and enables experiments that have truly never been possible before.

UNDER THE HOOD: IMAGESTREAM TECHNOLOGY AND OPERATION



ImageStream Core Technology

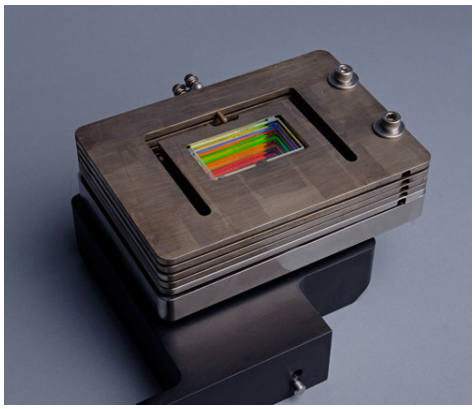
Ultimately, the key to successful 'imaging in flow' is keeping each moving cell in focus long enough to generate a useful image from its relatively dim fluorescent light emissions. Snap a picture too quickly (10 microseconds, for example) and the image will be too dim. Extend the exposure time, and the image blurs. The ImageStream overcomes this dilemma through the use of a uniquely designed six-channel CCD camera, a technology called Time Delay Integration and a precisely controlled fluidics system.



ImageStream 6-channel CCD Camera

Time Delay Integration, or TDI, was originally developed to provide high speed microscopic analysis for industrial process management. Applied in a flow cytometry system, TDI allows high speed, high resolution imaging of cells as they move in the fluid stream. The benefit, in brief, of TDI for the ImageStream is that cells moving in the flow stream may be imaged for almost 1000 times longer than a snapshot with no loss of resolution.

TDI is complemented in the ImageStream by a unique spectral decomposition element that is constructed from six dichroic mirrors, one for each channel in the instrument's CCD camera.



ImageStream dichroic mirror stack

Each of the six mirrors transmits light only of a closely defined spectral band; the sum of the bands covers the usable fluorescence emission spectrum. The set of mirrors in effect 'decomposes' a single image into six component images, providing multiple 'views' of every cell. The result is that the ImageStream generates, from every cell analyzed, six simultaneous images: brightfield, darkfield and up to four fluorescent images.

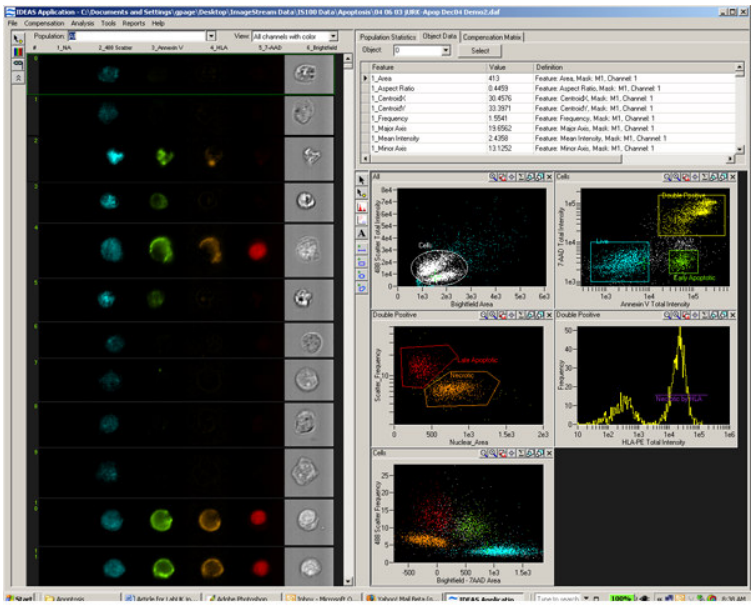
The powerful combination of TDI and spectral decomposition delivers high sensitivity, high resolution imagery from cells in flow. This imagery may then be analyzed using the ImageStream's powerful IDEAS® image analysis software.

Great Ideas: power and innovation in cell analysis.

The goal of the IDEAS software package is to allow users to make meaningful quantitative statements about the cells and populations they are studying in the ImageStream system. To do this – to address and quantify the true complexity of biological structures – the software must incorporate a very large number of descriptors, called 'features' in the IDEAS package. These include strictly morphological criteria such as cell size, aspect ratio and centroid, as well as more image-based criteria. In all, over 200 features are collected for every cell. Each feature provides the researcher a measured value about the cell under study. The entire feature set thus offers an immense flexibility and range in designing an analysis. In addition, the features themselves may be combined or modified algebraically, extending almost infinitely the ability of the feature set to address almost any morphologic evaluation.

In operation, the IDEAS package mirrors the two underlying technologies of the ImageStream system - microscopy and flow cytometry - and provides a link between them. One half of the

user interface displays cell images, while the other half displays the scatterplots, histograms and tables commonly used in flow cytometric analysis.



Cell Image Gallery Analytical Tools

The IDEAS User Interface

The two data formats – images and statistical representations – are fully linked. In IDEAS, every dot on a scatterplot is connected to a cell image. The user may click on a dot to see the corresponding image, or click on the image to find its location in a scatterplot. This highly heuristic correspondence makes it possible for the user to design and evaluate analytical strategies with real cell imagery, rather than through abstract representations only. The power of the IDEAS software opens up a wide range of novel applications for imaging in flow.

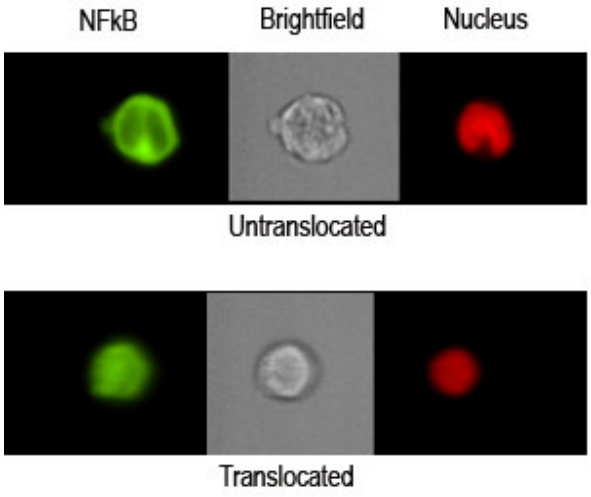
MAKING IT REAL: APPLICATIONS OF IMAGESTREAM TECHNOLOGY

Molecular Distribution

A conventional PMT-based flow cytometer measures signal intensity only – i.e., the amount of fluorescence emission from each probe bound to a given cell. In contrast, the ImageStream imaging flow cytometer measures not only signal intensity, but also the location of the signal on or in the cell. In many applications, the location of the signal is as important – or more important – than the amount of signal itself. One excellent example is in the study of nuclear translocation.

Movement of NFκB from Cytoplasm to Nucleus

The nuclear factor NFκB has been shown to be involved in the control of a variety of important cell processes by interacting with specific genes in the cell nucleus. Usually located in the cell cytoplasm, the protein migrates to the nucleus under the appropriate conditions. The fraction of total NFκB found in the nucleus gives an indication of how responsive the cell has been to a stimulus. In this example, the total *amount* of NFκB in the cell does not change when the cell is affected, but the *location* of the protein in the cell may change dramatically.

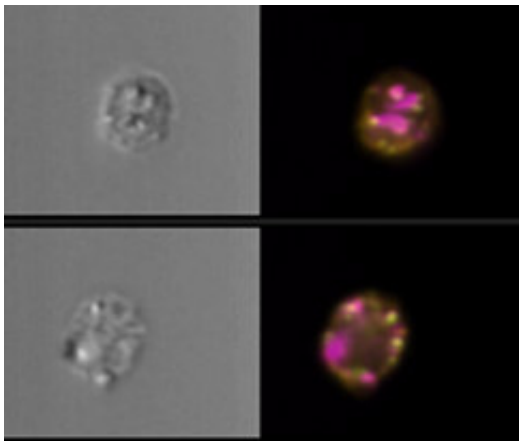


Imaging NFkB Translocation

By measuring signal intensity alone, it is impossible to distinguish stimulated cells from unstimulated cells. But with the ImageStream system, the two cell types appear distinctly different.

Intracellular Trafficking

In evaluating the mechanisms of action of antibody based therapeutics, it is often useful to study the ways in which target cells metabolize them. In the standard endocytic process, the drug, first bound to the cell surface, moves to endosomes and then to lysosomes. Each of these organelles can be located and tracked individually with fluorescent probes, as can the antibody itself.



Analysis of Molecular Trafficking

The ImageStream can readily identify which organelle the antibody drug is associated with at any given time. Here, as with the case of nuclear translocation of NF□B, the amount of the target molecule in the cell remains roughly the same during the process, but its location can change significantly. Determining the location of the antibody in the cell can be accomplished with the ImageStream, but would be impossible with a conventional flow cytometer.

Cell Classification

Many cell populations contain a diversity of cell types that can be distinguished by morphologic factors. A common and important example is the population of leukocytes (white cells) found in the blood. Evaluation of many disease states includes a differential analysis of the presence of neutrophils, basophils, lymphocytes, eosinophils and monocytes. On the ImageStream, these cell types may readily be distinguished using morphologic factors such as nuclear size and shape. No only does analysis on the ImageStream provide statistically significant numbers automatically, it allows the evaluation of additional parameters on the unused fluorescence channels.

Modes of cell death.

Cell death – clearly an important physiologic process – can occur either by necrosis or by a controlled, programmed process called apoptosis. On a conventional flow cytometer, it is all but impossible to tell apoptotic cells from necrotic cells. But with the morphologic evaluation available on the ImageStream, these two modes of cell death may be clearly distinguished.

A continually expanding range of applications.

The ability to quantitate morphological characteristics has proven valuable in other areas, as well. The formation of micronuclei, the progress of malarial infection, budding of yeast cells and pseudopod formation are all processes that involve substantial change in the shape of the cell or its components. Many pharmaceutical drugs also cause measurable changes to cell shape. By quantitating cell morphologies, the ImageStream system brings a high degree of statistical rigor to the analysis of these visual phenomena and many others like them. Conventional flow cytometry, as before, is powerless in this arena.

CONCLUSION

The ImageStream system from Amnis Corporation makes a remarkable and valuable contribution to the analysis of blood cells and other non-adherent cells. Through the innovative use of core technologies such as Time Delay Integration and Spectral Decomposition, and supported by a powerful and flexible image analysis software program, the ImageStream system brings the power of statistics to microscopy, the power of imaging to flow cytometry, and truly creates an entirely new way to think and work with cells in suspension.

This article was first published in Laboratory News, February 2006

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